

**Amendments to the Specification:**

Please replace the paragraph at page 1, lines 3-6, with the following amended paragraph:

This application is a divisional of U.S. Application No. 09/827,428 filed April 6, 2001, which is a continuation-in-part of U.S. Application ~~Serial~~ No. ~~09/599,487~~ 09/599,487, filed June 23, 2000, which claims priority from U.S. Provisional Application ~~Serial~~ No. 60/141,563, filed June 29, 1999, all of which are incorporated herein by reference in their entirety.

Please replace the paragraph at page 1, lines 14-16, with the following amended paragraph:

The invention relates to the fields of medicine and cell biology. More specifically, the invention relates to the fields of drug discovery and dermatology, particularly the biology of skin ~~pigmentation~~ pigmentation.

Please replace the paragraph at page 4, lines 1-12, with the following amended paragraph:

Thus, although P protein is known to be critical for the production of normal amounts of melanin in the skin, hair and eyes, the function of the P protein in this process has remained elusive. Instead, researchers have looked to other molecular targets for inhibition studies. For example, tyrosinase's well-characterized enzymatic activity, amenability to biochemical analysis, and pivotal role in melanogenesis ~~[[have]]~~ has made it an inviting target for inhibition studies (*see, e.g., Tasaka, K., et al., 1998, Meth. Find. Exp. Clin. Pharmacol.* 20:99-109; Iida, K., et al., 1995, *Planta Med.* 61:425-28; Reish, O., et al., 1995, *Am. J. Hum. Genet.* 57:127-32; Shirota, S., et al., 1994, *Biol. Pharm. Bull.* 17:266-69; Kameyama, K., et al., 1989, *Differentiation* 42:28-36). Researchers have also focused on the effects of intercellular signaling molecules on melanogenesis (*see, e.g., Furumura, M. et al., 1998, Proc. Natl. Acad. Sci. (USA)* 95:7374-78; Sakai, C., et al., 1997, *EMBO J.* 16:3544-52; McLeod, S.D. et al., 1995, *J. Endocrinol.* 146:439-47).

Please replace the paragraph at page 4, lines 24-31, with the following amended paragraph:

A number of products have been developed to effect a decrease in skin pigmentation. One such product contains hydroquinone, a ~~well-known~~ well-known active substance for skin de-pigmentation (*e.g.*, see U.S. Patent No. 6,139,854). However, hydroquinone can have serious side effects if applied over a long period of time. For example, the application of hydroquinone to skin may lead to permanent de-pigmentation, and thus to increased photosensitivity of the skin when exposed to ultraviolet light. For that reason, in some countries hydroquinone is only allowed to be used for skin de-pigmentation in limited concentrations, and, in other countries, the product is banned completely for this application.

Please replace the paragraph bridging pages 4 and 5, with the following amended paragraph:

A variety of other substances have been proposed for the control or inhibition of skin pigmentation. Almost all of these substances work by either bleaching existing pigment or preventing new pigment synthesis by inhibiting the activity of tyrosinase, the principle rate-limiting enzyme in the production of melanin. For example, U.S. Patent No. 6,123,959 describes the use of aqueous compositions comprising liposomes and at least one competitive inhibitor of an enzyme for the synthesis of melanin in combination with at least one non-competitive inhibitor of an enzyme for the synthesis of melanin. U.S. Patent No. 6,132,740 describes the use of certain resorcinol derivatives as skin lightening agents. WO 99/64025A1 describes compositions for skin lightening which contain ~~tyrosinase-inhibiting~~ tyrosinase-inhibiting extracts from dicotyledonous plant species indigenous to Canada. U.S. Patent No. 5,580,549 describes an external preparation for skin lightening comprising 2-hydroxybenzoic acid derivatives and salts thereof as inhibitors of tyrosinase. WO 99/09011A1 describes an agent for inhibiting skin erythema and/or skin pigmentation, containing at least one carbostyryl derivative and salts thereof. U.S. Patent Nos. 5,214,028 and 5,389,611 describes lactoferrin hydrolyzates for use as a ~~tyrosinase-inhibitory~~ tyrosinase-inhibitory agents.

Please replace the paragraph bridging pages 14 and 15, with the following amended paragraph:

Figure 4 is a graphic presentation of tyrosinase activity in transfected COS cells. COS cells transfected with a vector carrying a tyrosinase-encoding gene, or with a first vector carrying a tyrosinase-encoding gene and with a second vector carrying a P protein-encoding gene as in Figure 3, were treated with benztropine, imipramine, nitroquipazine, or left untreated, as in Figure 2. Cell extracts were prepared as in ~~Figure 3~~ Figure 3. The tyrosine hydroxylase activity of cell extracts was determined as in Figure 1 as a measure of tyrosinase activity. Column 1, untreated transfectants; Column 2, transfectants treated with benztropine; Column 3, transfectants treated with imipramine; Column 4, transfectants treated with nitroquipazine. Tyrosine hydroxylase activity is measured in cpm [<sup>3</sup>H]H<sub>2</sub>O/60 micrograms protein/hr. Cells co-transfected with a tyrosinase-encoding gene and a P protein-encoding gene (T + P) show a higher tyrosine hydroxylase activity than cells transfected with a tyrosinase-encoding gene alone (V + T) (column 1). This effect is not altered by incubation of cells in the presence of benztropine (column 2) or nitroquipazine (column 4). The presence of imipramine, however, abolishes the effect of P protein while appearing to have little effect on the activity in the cells with tyrosinase alone (column 3).

Please replace the paragraph at page 26, lines 6-28, with the following amended paragraph:

Another type of assay can be used to determine the presence or absence of the C-terminal portion of the tyrosinase protein. This assay depends, in part, on the discovery that melanogenic cells inhibited for melanogenesis (*e.g.*, by mutations or compounds that inhibit P protein function) contain and secrete a version of tyrosinase that lacks the C-terminal portion of tyrosinase, including its transmembrane domain and its ~~protein-sorting~~ protein-sorting signal. As explained above, this truncated form of tyrosinase nonetheless retains catalytic activity. In a non-limiting example of a method of screening based on this assay, melanogenic cells are grown or incubated in the presence of a test compound. An assay is selected that allows the length

and/or mass of tyrosinase protein to be determined. For example, Western blots or other immunohistochemical techniques using antibodies that recognize the N-terminal or central portions of the tyrosinase protein, or other standard molecular biological techniques useful for the determination of protein length or mass, can be performed on extracts of these cells and/or on their growth or incubation medium. Antibodies appropriate for these assays can be prepared using standard immunological techniques. *See, e.g.,* Harlow and Lane, 1988, *supra*. If the assay reveals the presence of a shorter or lower molecular weight form of tyrosinase, relative to similar cells grown or incubated under similar conditions but without the test compound, then the test compound inhibits melanogenesis. Alternatively, Western blots or other immunohistochemical techniques using antibodies recognizing the C-terminal portion of tyrosinase (*e.g.,* the anti-PEP7 antibody prepared as described in Jimenez *et al.*, 1991, *J. Biol. Chem.* 266:1147-1156) can be used in the assay. In these assays, a reduction in the amount of tyrosinase protein detected by the antibodies indicates that the test compound inhibits melanogenesis, because the truncated tyrosinase lacks the sequences recognized by the antibodies.

Please replace the paragraph bridging pages 26 and 27, with the following amended paragraph:

Both ~~full-length~~ full-length tyrosinase, and the truncated tyrosinase found in and secreted by melanogenic cells with inhibited or absent P protein, remain catalytically active when run on non-denaturing polyacrylamide gels. This observation is the basis, in part, of another assay for the truncated tyrosinase protein. Thus, melanogenic cells can be grown or incubated in medium containing a compound to be tested. Either the growth or incubation medium is collected, or cell extracts are prepared, and subjected to non-denaturing polyacrylamide gel electrophoresis. Smaller, more flexible proteins will migrate farther than larger proteins with more complicated three-dimensional structure. Filter paper or a membrane (*e.g.,* nitrocellulose) is soaked in L-DOPA and applied to the gel. Active tyrosinase in the gel converts L-DOPA into melanin, creating dark spots on the filter or membrane indicating the

location and, therefore, the relative size, of tyrosinase. If cells treated with the test compound produce two spots on the filter or membrane, wherein one spot indicates tyrosinase of the same size as produced by similar cells grown under similar conditions but without the test compound, and the other spot indicates tyrosinase of smaller relative size, then the test compound is a candidate for a compound that inhibits melanogenesis.

Please replace the paragraph at page 27, lines 8-29, with the following amended paragraph:

~~Full-length~~ Full-length tyrosinase in wild-type melanogenic cells with normal P protein function is found primarily in the insoluble fraction of cell extracts. To be released, it must be solubilized with a detergent (*e.g.*, Triton X-100™). In contrast, the smaller truncated version of tyrosinase in melanogenic cells with inhibited P protein function is found in vesicles in the soluble fraction. These observations are the basis, in part, of another assay that can be used to detect truncated tyrosinase in P protein-compromised cells. Thus, melanogenic cells are grown or incubated in medium containing a compound to be tested. The cells are harvested and can be subjected to a detergent phase separation to separate membrane-anchored proteins from soluble proteins. For example, the cells can be solubilized on ice in a buffer containing Triton X-114™ detergent. Insoluble contaminants can be spun out at 4°C. Then the supernatant, which contains solubilized proteins, is phase-separated at room temperature or elevated temperatures into a detergent phase and an aqueous phase. The ratio of tyrosinase in the detergent phase (which will contain tyrosinase proteins containing the C-terminal portion of the protein which anchors tyrosinase in the membrane) to tyrosinase in the aqueous phase (which will contain tyrosinase proteins which lack the C-terminal portion) is determined. Alternatively, cells are harvested and membranes disrupted by a freeze/thaw cycle or cycles. The disrupted cells are then separated into a soluble fraction and a membrane-bound, insoluble fraction. The ratio of soluble tyrosinase in the soluble fraction to insoluble, membrane-bound tyrosinase in the membrane fraction is determined. If cells treated with the test compound have higher levels of soluble tyrosinase than insoluble, membrane-bound tyrosinase than that from similar cells

grown under similar conditions but without the test compound, then the test compound is a candidate for a compound that inhibits melanogenesis.

Please replace the paragraph bridging pages 28 and 29, with the following amended paragraph:

Another consequence of inhibited melanogenesis can be the aberrant development of melanosomes. Wild-type melanogenic cells typically contain abundant, fully developed, darkly pigmented melanosomes. Such fully developed, darkly pigmented melanosomes are less abundant or absent in melanocytes inhibited for melanogenesis due to a mutation in the P protein-encoding gene when they are grown or incubated in medium containing low concentrations of tyrosine. Rather, these cells contain an unusually large number of immature melanosomes. This phenomenon is the basis, in part, for another assay that can be used. In a non-limiting example of a method of screening for compounds that inhibit melanogenesis that uses this type of assay, melanogenic cells are grown or incubated in medium containing a test compound. The number, size, shape, and/or color of the melanosomes in the cells is assayed. Such assays are well known in the art. For example, cells can be fixed and stained and examined using light microscopy. Alternatively, cells can be fixed, stained, sectioned, and examined using electron microscopy. Alternatively, cells can be fractionated using density centrifugation. Mature melanosomes are denser than immature melanosomes, and so can be separated from them on the basis of density using ~~well-known~~ well-known techniques. Cells treated with a test compound that have melanosomes that are altered in number, size, shape, and/or color compared to melanosomes from similar cells grown or incubated under similar conditions but without the test compound indicates that the test compound inhibits melanogenesis.

Please replace the paragraph bridging pages 45 and 46, with the following amended paragraph:

In an alternative embodiment, the P protein-encoding gene antisense nucleic acid of the invention is produced intracellularly by transcription from ~~[[an]]~~ a heterologous sequence. For example, a vector can be introduced *in vivo* such that it is taken up by a cell, within which cell the vector or a portion thereof is transcribed, producing an antisense nucleic acid (RNA) of the invention. Such a vector would contain a sequence encoding the P protein-encoding gene antisense nucleic acid. Such a vector can remain episomal or become chromosomally integrated, as long as it can be transcribed to produce the desired antisense RNA. Such vectors can be constructed by standard recombinant DNA technology methods known in the art. Vectors can be plasmids, viral vectors, or others known in the art as useful for replication and expression in mammalian cells. Expression of the sequence encoding the P protein-encoding gene antisense RNA can be regulated by any promoter known in the art to act in such cells. Such promoters can be inducible or constitutive, and can include but are not limited to those listed above.

Please replace the paragraph bridging pages 49 and 50, with the following amended paragraph:

The compounds useful according to the invention, and their pharmaceutically acceptable salts, are useful in the treatment of disorders of human pigmentation, including solar and simple lentigines (including age/liver spots), melasma/chloasma and ~~postinflammatory~~ post-inflammatory hyper-pigmentation. Such compounds reduce skin melanin levels by inhibiting the production of melanin, whether the latter is produced constitutively or in response to UV irradiation (such as sun exposure). Thus, some of the active compounds used in this invention can be used to reduce skin melanin content in non-pathological states so as to induce a lighter skin tone, as desired by the user, or to prevent melanin accumulation in skin that has been exposed to UV irradiation. They can also be used in combination with skin peeling agents (including glycolic acid or trichloroacetic acid face peels) to lighten skin tone and

prevent repigmentation. Other compounds useful according to the invention, and their pharmaceutically acceptable salts, are useful in the treatment of skin conditions where insufficient skin pigmentation is produced, or where the subject, for cosmetic purposes, simply wishes to develop a "sunless tan".

Please replace the paragraph at page 59, lines 9-17, with the following amended paragraph:

By the phrase "reducing skin pigmentation" is meant a detectable decrease in the amount of melanin in the skin, preferably causing a lightening of the skin as a result of a lowering of the amount of melanin synthesized *de novo*. The term "lowering" preferably refers to ~~[[a]]~~ about a 10% to about a 100% decrease in the amount of melanin synthesized *de novo*. More preferably, the term "lowering" refers to about a 25% to about a 100% decrease in the amount of melanin synthesized *de novo*. Most preferably, the term "lowering" refers to about a 50 to about a 100% decrease in the amount of melanin synthesized *de novo*. This lowering of melanin synthesized *de novo* is preferably visually distinguishable to the naked eye, *i.e.*, would not require the aid of a microscope or other such means to detect its occurrence.

Please replace the paragraph at page 67, lines 4-20, with the following amended paragraph:

The preferred oil-in-water emulsions comprise from about 0.05% to about 10%, preferably from about ~~[[I%]]~~ 1% to about 6%, and more preferably from about ~~[[I%]]~~ 1% to about 3% of at least one hydrophilic surfactant which can disperse the hydrophobic materials in the water phase (percentages by weight of the topical carrier). The surfactant, at a minimum, must be hydrophilic enough to disperse in water. Suitable surfactants include any of a wide variety of known cationic, anionic, zwitterionic, and amphoteric surfactants. See, ~~McCutcheon's Detergents and Emulsifiers~~, McCutcheon's Detergents and Emulsifiers, North American Edition (1986), published by Allured Publishing Corporation; U.S. Patent No. 5,011,681 to Ciotti *et al.*, issued April 30, 1991; U.S. Patent No. 4,421,769 to Dixon *et al.* issued



[[to]] December 20, 1983; and U.S. Patent No. 3,755,560. The exact surfactant chosen depends upon the pH of the composition and the other components present. Preferred are cationic surfactants, especially dialkyl quaternary ammonium compounds, examples of which are described in U.S. Patent No. 5,151,209 to McCall *et al.* issued [[to]] September 29, 1992; U.S. Patent No. 5,151,210 to Steuri *et al.* issued ~~to September~~ September 29, 1992; U.S. Patent 5,120,532; U.S. Patent 4,387,090; U.S. Patent 3,155,591; U.S. Patent 3,929,678; U.S. Patent 3,959,461; McCutcheon's, Detergents & Emulsifiers (North American edition 1979) M.C. Publishing Co.; and Schwartz, *et al.*, Surface Active Agents, Their Chemistry and Technology, New York: Interscience Publishers, 1949.